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ACID PHOSPHATASE ACTIVITY IN MELANOSOMES*

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In 1965, Seiji and Iwashita (1) found acid phophatase and adenosine triphosphatase, in addition to tyrosinase, the enzyme responsible for melanin formation, in a melanosome fraction isolated by density gradient centrifugation from Harding-Passey mouse melanoma. The biological significance and origin of these two enzymes have not yet been clarified. In this paper, the origin of acid phosphatase activity is studied.

Experimental mouse melanomas such as Harding-Passey and B-16 contain a significant number of phagocytes, and preparations obtained from these tissues by biochemical methods contain not only melanosomes that originate in melanocytes, but also melanosomes that were present in phagocytes. It is possible therefore that some of the melanosomes transferred to histiocytes may contain lysosomal enzymes adhering to melanosomes. The acid phosphatase found in the melanosome fraction is thought to originate from melanosomes in phagocytes. The question arises whether all of the acid phosphatase activity found in the melanosome fraction is derived from melanosomes in the phagocytes or whether the individual melanosomes in the melanocyte may contain acid phosphatase activity.

To answer this question, the acid phosphatase activity of melanosomes in the melanocyte of B-16 mouse melanoma was studied by means of electron microscopy and cytochemical staining methods.

MATERIALS AND METHODS

B-16 mouse melanomas used in the experiment reported here were serially transplanted in C-57 strain mice. The actively growing tumors were excised when their diameter reached 1.0 cm.

Small pieces of B-16 mouse melanoma were prefixed at 4° C for 2 hours in 6.25% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4. Then the tissues were rinsed and washed with the sodium cacodylate buffer containing 7.5% sucrose for two days at 4° C followed by incubation of the specimens at 37° C. The incubation medium contained lead ions to trap the phosphate ions released by the enzyme in the sections. The phosphate esters used were β-glycerophosphate in the medium of Gomori (2). The incubation medium was freshly prepared before use as follows: 100 ml of 0.12% $Pb(NO_3)_2$ in 0.05 M sodium acetate buffer, pH 5.0, containing 7.5% sucrose was mixed slowly with 10 ml of a 3% solution of sodium-β-glycerophosphate; this mixture was warmed up to 60° C and kept at this temperature for one hour, and then for a while at room temperature; the solution was then decanted to eliminate the fine precipitate which develops during preparation. The specimens were incubated at 37° C for 15 minutes, and rinsed twice for one minute each in cold 0.05 M sodium acetate buffer, pH 5.0, containing 7.5% sucrose and 4% formaldehyde. After the first rinse, the specimen was washed briefly with 2% acetic acid solution and followed by a second rinse.

Control experiments were performed in two ways: without substrate and with 0.01 M sodium fluoride in the incubation medium as an enzyme inhibitor.

After incubation, the tissues were postfixed in 1% buffered osmium tetraoxide for 2 hours. The tissue blocks were dehydrated in graded concentrations of alcohol and infiltrated and embedded in Epon 812. Sections were prepared on a Porter-Blum MT 2 ultramicrotome, and mounted on copper grids. The sections were stained with a 2% aqueous uranyl acetate solution, and examined in a Hitachi 11 B electron microscope. Micrographs were taken at a magnification of 20,000 and enlarged photographically.

The specimens examined were stained only with uranyl acetate in order to avoid staining any nonspecific precipitate which might occur in the process of lead staining, but acceptable morphological details of the cell were obtained.

OBSERVATIONS

The general aspects of the B-16 mouse melanoma cells were in accord with those described by others (3, 4). The melanoma melanocyte can be identified by the presence of premelanosomes and melanosomes which occur as individual granules in the cytoplasm and show varying degrees of melanization.

Many of the melanosomes have a single outer membrane, in which there are distinct arrays of parallel inner membranes along the long axis.

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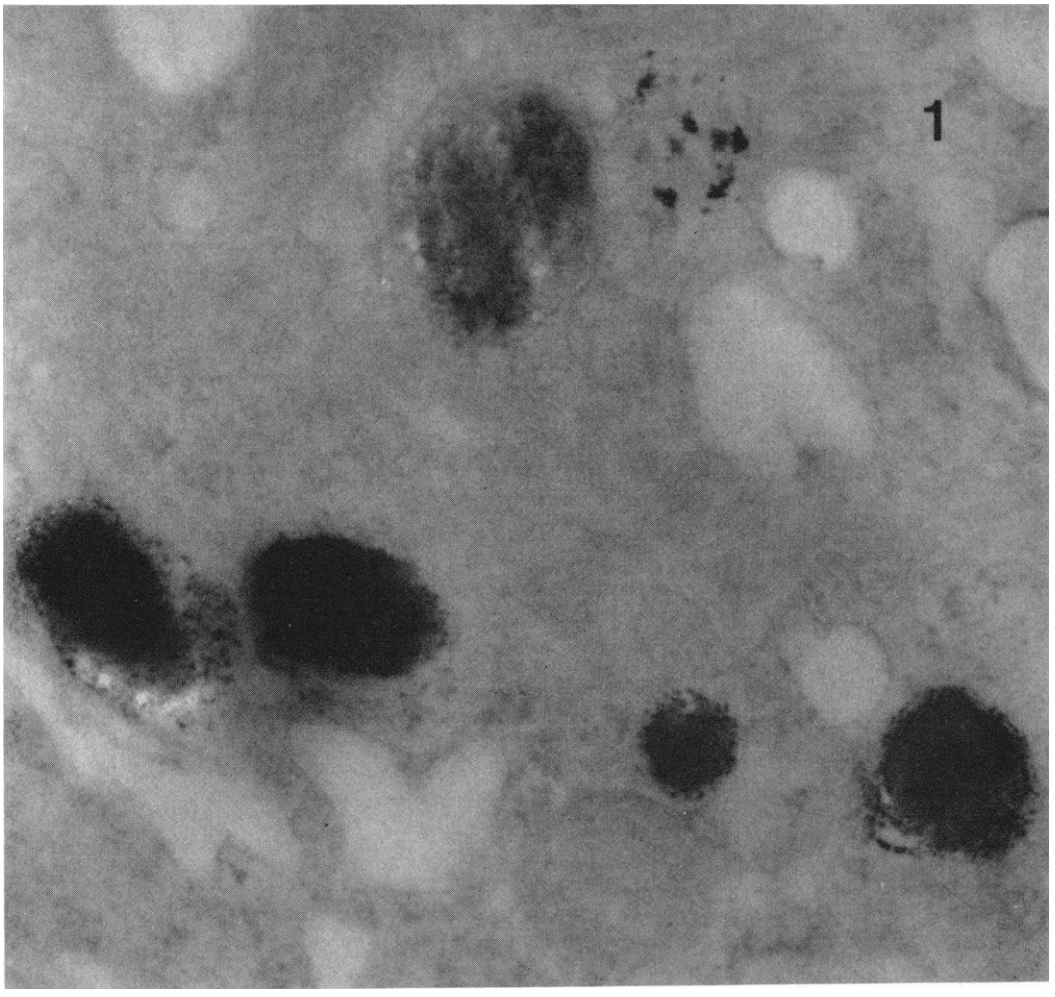

FIG. 1. Electron micrograph of part of a B-16 mouse melanoma melanocyte stained for acid phosphatase activity by Gomori's method. Fine or coarse opaque reaction products are seen to be present in variable amounts on the outer membranes of melanosomes. There is one lysosome which can be identified by the deposit of several coarse reaction products. × 80,000.

As melanin synthesis proceeds, the structure of the membranes is obscured, and in fully melanized melanosomes the inner structures can no longer be resolved.

Electron microscope examinations of the acid phosphatase reaction product on the melanoma melanocytes were carried out. Figure 1 shows part of a melanocyte in which there are five melanosomes and one lysosome. The melanization of these melanosomes is almost complete and the inner structure can no longer be resolved. Deposits of fine and coarse reaction products are present mostly on the outer membranes. The lysosome can be identified by the deposit of several large coarse reaction products.

In the control specimen shown in Figure 2, several melanosomes are present in the vicinity of the Golgi area of the melanocyte. No appreciable deposit of the reaction product is visible on the melanosomes. Figure 3 shows five melanosomes, four of which are in longitudinal section, and the fifth in cross section. Fine opaque reaction products are present in variable amounts on the inner membranes. The cross section shows that more reaction products are present on the outer membrane than the inner membrane. In

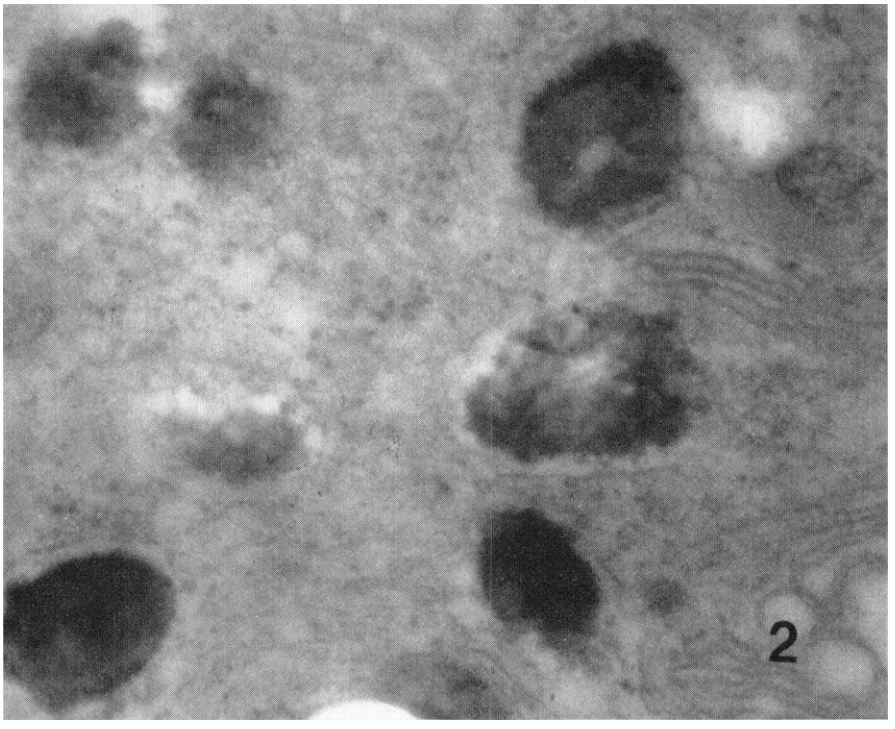

FIG. 2. A control for stained preparation which has been incubated in the staining medium for acid phosphatase activity in the presence of 0.01 M sodum fluoride. Several melanosomes are present in the vicinity of the Golgi area, but no reaction product is visible on the melanosomes. × 80,000.

this respect, Figure 4 shows a more detailed relationship between the reaction product and the membrane structure. Fine reaction products are distributed rather evenly on the outer membranes and coarse grains appear to line up along the inner membranes which run parallel to the long axis of the granule.

DISCUSSION

Acid phosphatase is one of the many hydrolytic enzymes that have been recognized in lysosomes (5), and is considered a reliable marker for histochemical identification of these organelles by the electron microscope (6, 7). An electron microscopic histochemical investigation was carried out on B-16 mouse melanoma in order to clarify the origin of acid phosphatase in the melanosome fraction isolated from mouse melanoma. B-16 mouse melanoma was used since the ultrastructure of the B-16 mouse melanoma melanosome is more discernible than that of the Harding-Passey mouse melanoma.

The presence of histochemically detectable acid phosphatase activity in the melanosomes in the melanocytes is clearly shown in Figures 1, 3 and 4. Melanosomes in various developmental stages in the melanocyte showed deposits of acid phosphatase reaction products in various amounts on their membranes. Fine or coarse opaque grains of reaction products are on both the outer and inner membranes, and these observations suggest that the more fully melanized melanosomes (Fig. 1), contain more deposits of reaction products. Interestingly, there seems to be a parallel relationship between melanization and the acid phosphatase activity of the melanosomes. Acid phosphatase may be present in the membranes of the melanosomes, which are not related to the site of melanin deposits.

No visible reaction product was found on the control specimens which were incubated in the reaction medium containing 0.01 M sodium fluoride (Fig. 2).

Mishima (8) found that acid phosphatase

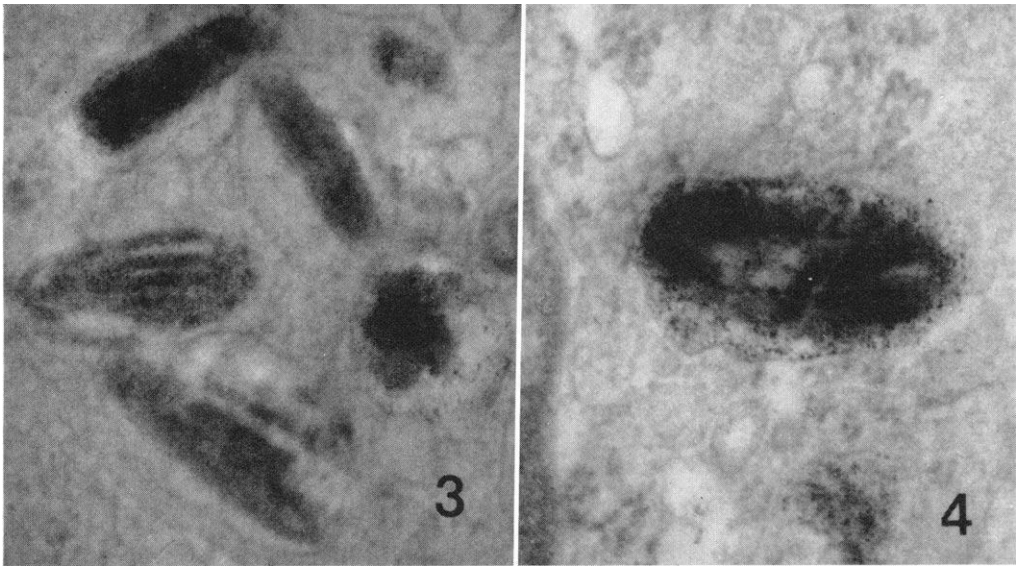

FIG. 3. Melanosomes in varying stages of melanization show the relationship between deposit of the reaction product and the membrane structure of melanosomes. Five melanosomes, four in longitudinal section and the fifth in cross section, are shown. Fine opaque reaction products can be seen on the inner membrane. The cross section shows more reaction product to be present on the outer membrane. × 80,000.

FIG. 4. Electron micrograph of a melanosome, in which melanization is fairly advanced. Fine reaction products are seen to be distributed evenly on the outer membrane and coarse grains appear to line up along the inner membranes. × 80,000.

activity was present on aggregate forms of melanosomes within melanophages in Dubreuilh's precancerous melanosis. Fitzpatrick *et al.* (9) thought that the presence of acid phosphatase in melanosome fractions isolated from mouse melanoma was due to the melanosomes from phagocytes which might contain lysosomal enzymes. They examined melanosome preparations obtained biochemically from a monolayer of melanocytes, the retinal pigment epithelium of 10-day-old white leghorn chick embryos, but were unable to find acid phosphatase activity. Electron microscopic histochemical observations now under way in our laboratory confirmed their findings. According to the previous interpretation reported herein, it may be possible that the melanization of melanosomes in 10-day-old chick embryos is not fully developed, thus producing less acid phosphatase activity.

During these histochemical observations, a histochemically detectable acid phosphatase activity in the Golgi area of the melanoma melanocytes was observed. Although the mechanism of how melanosomes acquire acid phosphatase is at present not entirely clear, it is probably possible to assume that melanosomes in various developmental stages merge with primary lysosomes arising from the Golgi area (7).

Recently, it has been theorized that melanin granules are broken down not only by macrophages but also in melanocytes (4, 10) and epidermal cells (11). There is a possibility, therefore, that such lysosomes may play a certain role in the degradation process of melanin granules.

SUMMARY

A combined cytochemical and electron microscopic demonstration of acid phosphatase activity of melanosomes in melanocytes was carried out on B-16 mouse melanoma. The presence of histochemically detectable acid phosphatase activity in the melanosomes was clearly demonstrated. The origin and biological significance of acid phosphatase in the melanosome was discussed.

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